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Transgenic rats carrying human c-Ha-*ras* proto-oncogenes are highly susceptible to *N*-methyl-*N*-nitrosourea mammary carcinogenesis

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A rat line carrying three copies of the human c-Ha-*ras* proto-oncogene, including its own promoter region, was established and designated Hras128. Expression of the transgene was detected in all organs examined from Hras128 rats by northern blot analysis. To examine its influence on susceptibility to *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis, female rats were treated with 50 mg/kg MNU i.v. at 50 days of age. All 22 Hras128 transgenic rats rapidly developed multiple and large mammary carcinomas within as little as 8 weeks after MNU treatment (14.1 tumors/rat, average diameter 16.4 mm). In contrast, 24 non-transgenic littermates developed no or only small tumors (0.46 tumors/rat, average diameter 7.4 mm) within this period. PCR–restriction fragment length polymorphism (RFLP) analysis and direct sequencing for the transduced human c-Ha-*ras* proto-oncogene indicated that 38 out of 44 tumors (86.4%) contained cells with mutations at codon 12 in exon 1. However, the signal densities of the mutated bands observed in the RFLP analyses revealed the presence of mixed populations of mutated and non-mutated cells in the tumors, the latter being in the majority. PCR–single strand conformation polymorphism analysis detected no mutations in codons 12 or 61 of the endogenous rat c-Ha-*ras* gene of Hras128 rat tumors. The results thus indicate that rats carrying the transduced human c-Ha-*ras* proto-oncogene are highly susceptible to MNU-induced mammary carcinogenesis and that this is not primarily due to mutations of the transgene or endogenous c-Ha-*ras* gene.

Introduction

Transgenic mice provide us with good animal models for many diseases and are widely used for analysis of various gene functions. In the field of chemical carcinogenesis, transgenic mice harboring the human c-Ha-*ras* proto-oncogene (the rasH2 mouse) (1,2), v-Ha-*ras* transgenic mice (TG.AC mice) (3), *pim-1* transgenic mice (4) and *p53* knockout mice (3) have been shown to be susceptible to tumor induction by certain carcinogens. In transgenic mice harboring the human c-Ha-*ras* proto-oncogene, more rapid onset and higher incidences of malignant tumors of skin, lung and forestomach than in non-transgenic mice were observed after treatment with various carcinogens (1,2).

For studies of chemical carcinogenesis, however, rats rather than mice are more frequently used, for various reasons. For example, in the liver a variety of enzyme-altered focal lesions have been studied for their relevance to carcinoma development (5–7) and some have been utilized as markers for early detection of preneoplastic lesions (8–11). In contrast, no equivalent marker lesions in mouse liver are available. Furthermore, mammary cancers in rats can be induced by MNU administration without the involvement of a viral etiology. However, only limited types of transgenic rats have been developed to study carcinogenesis. Rats containing an albumin promoter fused to the simian virus 40 T antigen gene have been used to investigate glutathione *S*-transferase (GST)-P expression in preneoplastic foci in the liver induced by the transgene (12) and another transgenic rat containing the GST-P promoter fused to the chloramphenicol acetyltransferase gene has been employed to study regulation of GST-P transcripts in rat liver carcinogenesis (13,14).

We have generated transgenic rats using the same gene construct used for generation of human c-Ha-*ras* proto-oncogene transgenic mice (15), which has no mutations in the protein coding regions and no ability to transform NIH 3T3 cells (16). In order to determine their susceptibility to mammary carcinogenesis, human c-Ha-*ras* proto-oncogene transgenic rats (Hras128 rats) were treated with *N*-methyl-*N*-nitrosourea (MNU), which is known to induce mammary carcinomas when applied i.v. (17). We show here that human c-Ha-*ras* proto-oncogene transgenic rats are highly susceptible to MNU-induced mammary carcinogenesis and that the susceptibility is not primarily due to mutation of the transduced human c-Ha-*ras* proto-oncogene.

Materials and methods

Transgenic rat

Sprague–Dawley rats (Clea Japan Inc., Tokyo, Japan) were used for the initial production of founder animals of transgenic rats. The DNA construct utilized for the transgenic rats has been previously described (16) (Figure 1). A 6.8 kb *Bam*HI fragment of the human c-Ha-*ras* proto-oncogene with its own promoter region eluted from agarose gel was purified using a Qiaex II Gel Extraction Kit (Qiagen, Hilden, Germany) and injected into pronuclei of a total of 1145 rat embryos collected from superovulated prepubescent Sprague–Dawley female rats mated with males of the same strain. Techniques used for generation of transgenic rats were essentially similar to those commonly used for transgenic mice (18).

Of 211 potential transgenic rats screened, two male rats were shown by PCR and Southern blotting to carry the transgene. These rats gave rise to transgenic offspring according to Mendelian genetics. Subsequent matings have been carried out between the transgenic and non-transgenic Sprague–Dawley rats to maintain rats heterozygote for the transgene.

PCR and Southern blotting

DNA samples from rat tails were obtained by the protenase K/phenol/chloroform method. PCR was performed using AmpliTaq Gold (Perkin Elmer, NJ) and human c-Ha-*ras* exon 2-specific primers, hHras2F (5′-AGCCCTGTCCTCCTGCAGGAT-3′) and hHras2R (5′-GGCCAGCCTCAC-GGGGTTCA-3′), which amplify a 218 bp fragment of human c-Ha-*ras*.

Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; GST, glutathione *S*-transferase; MNU, *N*-methyl-*N*-nitrosourea; RFLP, restriction fragment length polymorphism; SSCP, single strand conformation polymorphism.

Fig. 1. Structure of the human c-Ha-*ras* proto-oncogene used to generate transgenic rats. It contains its own promoter region in the 5′-region, a single nucleotide change in the last intron (asterisk) and the putative enhancer element of the 3′-downstream repeated sequence. B, *Bam*HI; S, *Sac*I; X, *Xba*I. Boxes marked I–IV indicate the coding exons (see ref. 16).

DNAs were digested by the restriction enzymes *Hin*dIII, *Xho*I, *Xba*I, *Eco*RI and *Sac*I and Southern blots were probed with a ^{32}P-labeled *Sac*I fragment of the human c-Ha-*ras* gene. The number of copies of the transgene was determined from the restriction enzyme cutting pattern and the amount of hybridization signal in 10 μg of genomic DNA, in comparison with signals for known c-Ha-*ras* gene amounts (Figure 2). Signals were measured with a BAS2000 image analyzer (Fuji Film Co. Ltd, Tokyo, Japan).

Northern blotting

Total RNAs were extracted by the acid guanidinium thiocyanate/phenol/chloroform method from multiple normal organs and mammary tumors of the transgenic and non-transgenic rats and 10 μg aliquots were loaded onto 1% agarose gels, electrophoresed and transferred to nylon membranes (Hybond-N$^+$; Amersham, Arlington Heights, IL). A human c-Ha-*ras* proto-oncogene mRNA-specific oligonucleotide probe (5′-GGGGTTCCGGTGGCATTTGG-3′) was labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (TaKaRa, Otsu, Japan) and hybridized to mRNA on the membrane. A cDNA probe for GAPDH was also hybridized as a control for the loaded amounts of RNA.

Carcinogenesis study

A total of 22 transgenic and 24 littermate non-transgenic female rats were treated with 50 mg/kg body wt MNU (Sigma-Aldrich Japan, Tokyo, Japan) injected into the tail vein at 50 days of age. Numbers and sizes of palpable mammary tumor masses were subsequently recorded. After 8 weeks, rats were killed and all mammary lesions were removed for histological evaluation and mutation analysis of the transduced human c-Ha-*ras* proto-oncogene and the endogenous rat c-Ha-*ras* gene.

Restriction fragment length polymorphisms (RFLPs) and direct sequencing

In preliminary experiments, we found that the particular single base substitution GGC→GAC of codon 12 of the transduced human c-Ha-*ras* proto-oncogene, most frequently found in rasH2 mice (1), could not be detected by the PCR–single strand conformation polymorphism (SSCP) technique. Therefore, we applied restriction fragment length polymorphism (RFLP) analysis to screen mutations in the transgene. A 167 bp fragment was amplified with primers hHras1F (5′-GCAGGCCCCTGAGGAGCGAT-3′) and hHrasIRN (5′-AGCA-GCTGCTGGCACCTGGA-3′) at an annealing temperature of 60°C for 35 cycles. When the DNA fragments was digested with *Msp*I, DNA of wild-type sequence (GGC) was cleaved to 117 and 50 bp, but those with base substitutions of the first and second G residues of codon 12 were not. Similarly, mutations in codon 61 were detected by the presence of an *Alw*NI-resistant band. An *Alw*NI site was generated by a mismatched primer (H61/2A2, 5′-CGCATGGCGCTGTACAGCTC-3′). For the first round PCR, 218 bp fragments of exon 2 were amplified by primers hHras2F and hHras2R at an annealing temperature of 63°C for 35 cycles and then diluted 100 times with water and used as templates for the second round PCR using primers hHras2F and H61/2A2 at an annealing temperature of 60°C for 35 cycles. DNA fragments with the wild-type sequence (CAG) was cleaved into 93 and 17 bp by *Alw*NI. The fragments with codon 61 mutants were not cut by this enzyme and gave a 110 bp band.

To investigate the detection sensitivity of the RFLP analyses, 100 times diluted PCR fragments containing exon 1 of the human transgene from DNA of normal liver of transgenic rats and from T24 cells (for exon 1), generated using primers hHras1F and hHras1RN, or DNA of normal liver of the transgenic rats and from plasmid pSK2 (for exon 2), generated using primers hHras2F and hHras2R, were mixed in different serial concentrations from 1:1 to 1:100 and used as templates for PCR following enzyme digestion. Digested samples from each reaction were electrophoresed in 2 or 4% agarose gels. When bands of DNA fragments resistant to digestion were visualized, the fragments were amplified with hHras1F and hHras1R for analysis of codon 12 and then directly sequenced using ^{32}P-end-labeled upper primers for each amplification (TaKaRa).

For positive controls for detection of mutations in codons 12 and 61 of the c-Ha-*ras* human proto-oncogene, DNA from T24 cells (GGC→GTC in codon 12) (19) (JCRB0711; Health Science Research Resources Bank, Japan Health Sciences Foundation) and plasmid pSK2 (CTG→CAG in codon 61) (20) (CO 001; Health Science Research Resources Bank, Japan Health Sciences Foundation), respectively, were used.

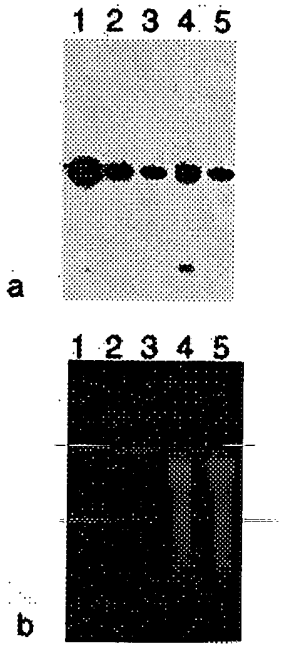

Fig. 2. (**a**) Southern blotting analysis. *Sac*I-digested plasmids containing 10 (lane 1), two (lane 2) or one (lane 3) copy of the transgene per 10 μg rat genomic DNA were assessed as controls along with 10 μg of Hras128 (lane 4) and Hras102 (lane 5) liver DNA digested with *Sac*I. Hybridization was performed with the *Sac*I fragment of the transduced human c-Ha-*ras* proto-oncogene. Intensities of bands (2.9 kb) were determined with a BAS 2000 image analyzer which revealed that Hras128 rats have three copies of the transgene and Hras102 have one. (**b**) Ethidium bromide staining of the agarose gel before Southern blotting, showing equal amounts of DNA (10 μg) loaded in lanes 4 and 5.

PCR–SSCP analysis

To screen possible mutations in the endogenous rat c-Ha-*ras* gene, exons 1 and 2 of the gene were analyzed by the PCR–SSCP technique (21). Pairs of primers were designed to amplify each exon separately. Primers for exon 1 were rHras1F (5′-GCGATGACAGAATACAAGCT-3′) and rHras1R (5′-GAGCTCACCTCTATAGTGGG-3′) and for exon 2 were cHras2IF (5′-CTGCAGGATTCCTACCGGAA-3′) and cHras2IR (5′-CACCTGTAC-TGGTGGATGTC-3′). PCR was performed using AmpliTaq Gold (Perkin Elmer, NJ) and ^{32}P-end-labeled primers at annealing temperatures of 51°C for exon 1 and 55°C for exon 2 and products were analyzed in 5% non-denaturing polyacrylamide gels (49:1) with or without 5% glycerol at 4 or 20°C. A small area (~1 mm^2) of the gel corresponding to the position of each mobility shifted band from normal tissue was cut out. The gel pieces were immersed in 20 μl water and heated at 80°C for 15 min. Then, this solution was used for PCR using the same primers as for PCR–SSCP analysis and then directly sequenced with ^{32}P-end-labeled upper primers for the PCR and a *Taq* cycle sequencing kit (TaKaRa).

Subcloning and sequencing analysis

Exon 1 of transgenes from four independent mammary carcinomas was amplified using primers hHras1F and hHras1R. PCR products were subcloned into pGEM-T Easy vectors (Promega Corp., Madison, WI) and transformations were performed using JM109 competent cells (Toyobo Inc., Tokyo, Japan). Around 50 plasmid DNAs having an insert were isolated from single colonies for each tumor and sequenced using a Cy5-labeled M13 universal primer, ThermoSequenase fluorescent labeled primer cycle sequencing kits and ALF express DNA sequencers (Amersham Pharmacia Biotech, Little Chalfont, UK).

Results

Generation of human c-Ha-ras proto-oncogene transgenic rats

Injection of the human c-Ha-*ras* proto-oncogene DNA construct (Figure 1) into pronuclei of a total of 1145 rat embryos gave rise to 211 potential transgenic rats, and two founder rats were obtained. Southern blotting analysis revealed that one

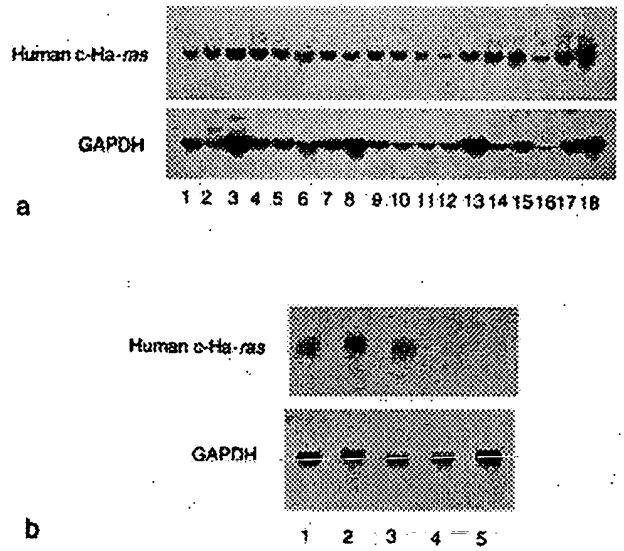

Fig. 3. (a) Northern blot analysis for human c-Ha-*ras* in various tissues of Hras128 rats. Lane 1, uterus; lane 2, skin; lane 3, muscle; lane 4, bladder; lane 5, ovary; lane 6, kidney; lane 7, large intestine; lane 8, small intestine; lane 9, glandular stomach; lane 10, forestomach; lane 11, spleen; lane 12, liver; lane 13, heart; lane 14, lung; lane 15, thymus; lane 16, salivary gland; lane 17, cerebellum; lane 18, cerebrum. A 1.1 kb mRNA from the transgenes was detected in all tissues examined. (b) Northern blot analysis for human c-Ha-*ras* in mammary gland tissue from Hras128 rats and non-transgenic rats. Lanes 1–3, RNA samples from Hras128 rats; lanes 4 and 5, RNA samples from non-transgenic rats. A 1.1 kb mRNA from the transgene(s) was detected in mammary tissue from the transgenic rats, but not from non-transgenic rats. GAPDH was demonstrated in the same filter to confirm the presence of RNA.

line had three copies and the other had one copy of the transduced gene (Figure 2). The former three-copy gene was transmitted to the next generations stably and its mRNA expression was detected in all organs examined (Figure 3a), including the mammary gland (Figure 3b). The rat strain was named Jcl/SD-TgN(HrasGEN)128Ncc (Hras128). However, expression of the transgene in the other line of rats with one copy (Hras102) was no longer detectable after two generations. Therefore, only the Hras128 rats were used for the following studies.

MNU-induced mammary carcinogenesis study

Hras128 transgenic rats proved highly susceptible to induction of mammary carcinogenesis by treatment with MNU. Five weeks after a single injection of MNU, multiple mammary tumors could be easily detected in almost all mammary organs by palpation in Hras128 rats, whereas no tumors were present in non-transgenic littermates of the same age. By the end of week 8, all Hras128 transgenic rats developed large multiple tumors throughout each of the 12 mammary glands with a progressively moribund condition (Figure 4). Therefore, the experiment was terminated at this time point. Data for tumor incidence and size (mean values of maximum diameter of respective tumors) are summarized in Table I. Only seven out of 24 (29.2%) non-transgenic littermates treated with MNU had tumors of small size. The histological appearance of all tumors was solid tubular or papillary tubular adenocarcinomas (Figure 5). A high expression of the transgene in the mammary carcinomas was confirmed by northern blot analysis (Figure 6). No other macroscopic or microscopic lesions were observed in either transgenic or non-transgenic rats. Several rats from

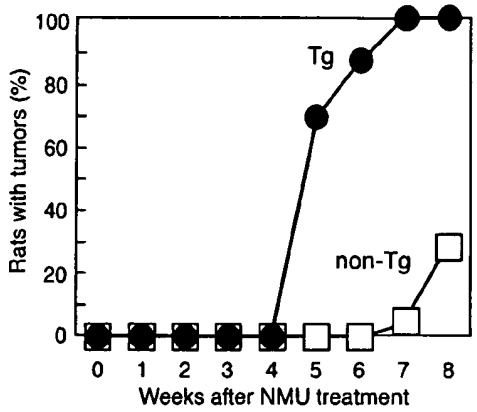

Fig. 4. Periodic observation of palpable mammary tumors in Hras128 rats and non-transgenic rats treated with MNU at 50 days of age. Closed circles, Hras128 rats (Tg); open squares, non-transgenic rats (non-Tg). Large and multiple mammary tumors developed in 100% of Hras128 rats whereas tumors were small and only found in 29.2% of non-transgenic rats.

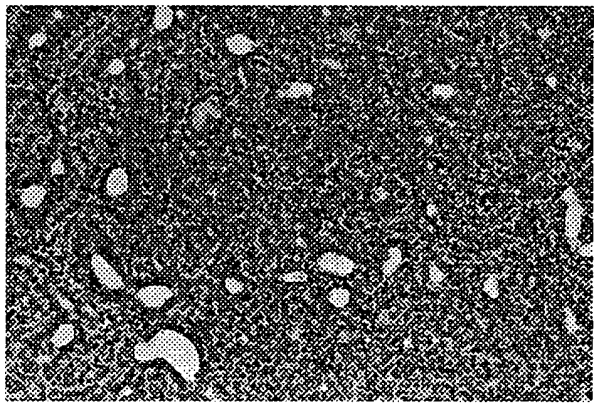

Fig. 5. Representative histological appearance of mammary tumors induced in Hras128 rats by MNU, diagnosed as solid tubular adenocarcinomas. Hematoxylin and eosin staining, ×100.

Table I. Quantitative data for mammary tumors induced by MNU in human c-Ha-*ras* proto-oncogene transgenic rats

Strain	Experimental period (weeks)	No. of rats	No. of tumor bearing rats (%)	No. of tumors per rat	Tumor size (mm)[a]
Hras128	8	22	22 (100)[b]	14.1 ± 6.42[c]	16.4 ± 9.00[c]
Non-Tg	8	24	7 (29.2)	0.46 ± 0.93	7.45 ± 6.02

[a]Maximum diameter.
[b]Significantly different by χ^2 test at $P < 0.001$.
[c]Significantly different by Mann–Whitney U-test at $P < 0.001$.

the other transgenic line (Hras102), carrying one copy of the same transgene, which is not transmitted to the third generation, also showed high susceptibility to MNU induction of mammary tumors (data not shown). Without carcinogen exposure, no tumors in the transgenic rats were noted within the experimental period.

Mutation analysis of the transduced human c-Ha-ras proto-oncogene by RFLPs

Thirty-eight out of 44 tumors were shown to have *Msp*I-resistant bands of DNA fragments amplified from the region carrying exon 1, indicating the presence of cells with mutations

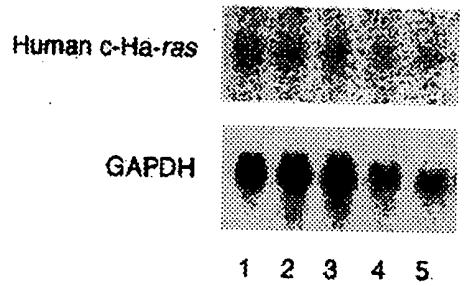

Fig. 6. Northern blot results for human c-Ha-*ras* in mammary carcinomas (lanes 1–3) and normal mammary tissue (lanes 4 and 5) of Hras128 rats. Transgene expression is apparent in the mammary carcinomas although comparison of levels with those in normal tissue (ducts) is difficult because the latter is a mixture of a small amount of mammary ducts, fatty and other soft tissue components.

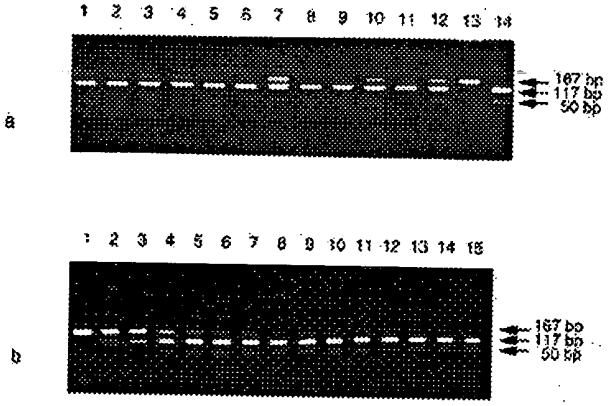

Fig. 7. Representative results of RFLP analysis for codon 12 (**a**) and the dilution standard of DNA (**b**). (a) Lanes 1–12, mammary tumors induced in Hras128 rats; lane 13, T24; lane 14, normal liver. In lanes 1–12, *Msp*I-resistant bands are visible but faint, which indicates that all tumors have mutations but that the mutant cell populations are not a major component. (b) Serial dilution of DNA for codon 12. Ratio of mutant/wild-type sequences: lane 1, 1/0; lane 2, 1/1; lane 3, 1/2; lane 4, 1/5; lane 5, 1/10; lane 6, 1/20; lane 7, 1/30; lane 8, 1/40; lane 9, 1/50; lane 10, 1/60; lane 11, 1/70; lane 12, 1/80; lane 13, 1/90; lane 14, 1/100; lane 15, 0/1. Mutant bands are visible even at the ratio of 1/100 in lane 14.

Table II. c-Ha-*ras* mutations in MNU-induced mammary tumors

Strain	No. of tumors examined	Transduced human c-Ha-*ras* proto-oncogene exons[a]				Rat c-Ha-*ras* gene exons[b]	
		1	2	3	4	1	2
Hras128	44	38[c] (86.4%)	0	0	0	0	0
Non-Tg	21					6[d] (28.6%)	0

[a]From both RFLP and SSCP analyses. 1, codon 12; 2, codon 61.
[b]From SSCP analysis.
[c]Thirty-four GGC→GAC; three GGC→GTC; one GGC→AGC.
[d]Six GGA→GAA.

in codon 12 of the transgene (Figure 7a and Table II). The density of the signals for mutant bands varied among the samples, two being high and 36 tumors being moderate to low. The dilution study for the evaluation of sensitivity of this RFLP analysis using the liver tissue of normal rats and mutant (T24) DNA fragments indicated that one mutant out of 100 wild-type sequences could be detected by this RFLP analysis. Based on the comparison of the signal density of bands in the

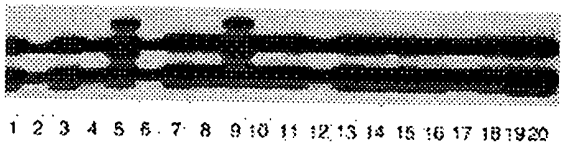

Fig. 8. Representative results of SSCP analyses with 5% glycerol at 20°C for rat c-Ha-*ras* exon 1. Lanes 1–10, PCR products for mammary tumors induced in non-transgenic rats; lanes 11–20, PCR products for mammary tumors induced in Hras128 rats. Lanes 5 and 9 demonstrate mobility shifted bands indicating the presence of mutation(s) in rat c-Ha-*ras* exon 1.

Table III. Mutation frequency analysis by PCR: subcloning of exon 1 of the transgene and sequencing

Tumor name	No. of clones	No. of mutant clones	Mutant sequence[a]
758M	51	6	AGC (codon 12)
761G	47	1	GAC (codon 12)
772B	47	0	
773H	51	3	GAC (codon 12)

[a]The wild-type of codon 12 is GGC.

DNA dilution study as shown in Figure 7b, it was estimated that tumors with a high density mutated band (for example Figure 7a, lane 7) contained ~10%, those with moderate density bands (lanes 3–5, 10 and 12) 2–5% and those with the low density band (Figure 7b, lanes 1, 2, 6, 8, 9 and 11) 1–2% cells carrying mutant PCR fragments, respectively. Similarly, with RFLP analyses performed for exon 2, no mutations were detected in any of the 44 tumors (Table II). Direct sequencing of low density bands of codon 12 as shown in Figure 7a revealed that 34 tumors contained GGC→GAC, three GGC→GTC and one a GGC→AGC mutation, respectively.

Mutation analysis of the endogenous rat c-Ha-ras genes in the mammary tumors by PCR–SSCP

Possible mutations in exons 1 and 2 of the rat endogenous c-Ha-*ras* gene were examined by PCR–SSCP analysis. Using specific primers for exon 1 of the rat gene, only two bands appeared on the gels. With common primers (cHras2IF and cHras2IR) for c-Ha-*ras* exon 2 of both human and rat, four bands were detected. Human- or rat-specific bands were identified by signals from normal DNA of the transgenic and non-transgenic rats. Tumors from non-transgenic rats demonstrated GGA→GAA mutations in six out of 21 (28.6%) in codon 12 of the endogenous c-Ha-*ras* gene on PCR–SSCP analysis followed by direct sequencing (Figure 8 and Table II). No mutation was detected in codon 61 of exon 2.

Subcloning and sequencing analysis

PCR amplification of DNA fragments carrying exon 1 taken from four tumors (named 758M, 761G, 772B and 773H), subcloning of the fragments using a plasmid vector and sequencing of individual clones from ~50 colonies/tumor revealed that six out of 51 clones from the 758M tumor carried mutations, all exhibiting a GGC→AGC mutation in codon 12. Similarly, a GGC→GAC mutation was observed in one out of 47 from 761G and three out of 51 from 773H colonies. All 47 clones from the 772B tumor had only the wild-type sequence, although RFLP analysis had indicated the presence of a GAC mutation in minor populations (Table III).

Discussion

The present study revealed that Hras128 transgenic rats carrying three copies of the human c-Ha-*ras* proto-oncogene, the

same gene as used to establish transgenic mice (1,2), responded to a single i.v. injection of MNU (50 mg/kg) by developing multiple, large sized mammary carcinomas at 100% incidence within as short a period as 8 weeks. The earliest palpable tumors appeared at week 5, rapidly increasing their size and number up to 14.1 tumors/rat, accompanied by a progressive cachexic condition. This is the first report of such large mammary carcinomas developing within an 8 week period after a single carcinogenic treatment in the rat. The results clearly indicate that expression of the transgene is associated with marked enhancement of susceptibility to MNU mammary carcinogenesis.

Transgenic mice prepared using the same DNA construct of the human c-Ha-*ras* proto-oncogene were reported to have a high susceptibility to forestomach, skin and lung carcinogenesis. It was therefore proposed that these mice could be candidates for medium-term carcinogenicity testing (2). However, since the proposed experimental period for mice, 6 months (22–24), is far longer than would be necessary with human c-Ha-*ras* proto-oncogene transgenic rats, the transgenic rats could be used as a good short-term screening model to detect carcinogens or compounds with inhibitory effects on carcinogenesis after appropriate modification (25).

In an earlier report, almost all forestomach tumors of human c-Ha-*ras* proto-oncogene transgenic mice induced by i.p. injection of MNU had a mutation of the transduced human c-Ha-*ras* proto-oncogene in codon 12 (GGC→GAC) (1). The transgenic mice also proved highly susceptible to 7,12-dimethylbenz[*a*]anthracene (DMBA) induction of lung and forestomach tumors in which mutations of the transduced human c-Ha-*ras* proto-oncogene in codon 61 (CAG→CTG) were frequently observed (26). Furthermore, no mutations in the endogenous mouse c-Ha-*ras* gene were detected, clearly indicating that the transgene is a target of carcinogens.

In the non-transgenic rat, mammary tumors induced by i.v. injection of MNU are known to have a high incidence of mutations in codon 12 of the c-Ha-*ras* gene (GGA→GAA) (17,27). However, other studies indicated that mammary tumors induced by DMBA exhibit a different mutation (A→T transversions at codon 61) (28). It is thus possible that the type of c-Ha-*ras* mutation is dependent on the inducing carcinogen. Another possibility is that carcinogens provide some selective environment for pre-existing mutations to persist, for example, specific G→A transversion in MNU-treated cases (29,30). The relatively low incidence (28.6%) of mutations of the endogenous c-Ha-*ras* gene in non-transgenic rat tumors may be linked to the short duration of the current experiment as compared with other studies where 50–92% incidences were noted (17,27).

Although the incidence of mammary carcinomas carrying a transduced human c-Ha-*ras* proto-oncogene mutation was relatively high, the actual mutated tumor cell population within the respective tumor tissue can be considered minor from the density of *Msp*I-resistant bands by RFLP analysis of the transgene codon 12 (Figures 6 and 7). This could be confirmed by subcloning and direct sequencing analysis of tumor tissue randomly taken from different rats (31). Since signals from the labeled mutated transgene DNA were diluted by co-existing normal cells as contaminants, estimation of the mutated to non-mutated cell ratio could be done by estimating the population of normal cells. For example, if the DNA examined had 20% (at the most) normal cell contamination as estimated by their histological appearance, as shown in Figure 5 (mammary tumors in these transgenic rats usually contain a low percentage of normal cells), and if only one of the three copies of the transgene was mutated, the population of mutated cells in the 758M, 773H and 761G subclones would be 44, 22 and 8%, respectively (number of mutated clones/number of total clones×3 copies) (see Table III). Accordingly, the mutated cell population was <50% of the total number of tumor cells in all cases, precluding a major role in the enhancement of susceptibility to MNU carcinogenicity. It should be noted that, based on the comparison of signal density bands, a large majority of mammary tumors in non-transgenic rats were considered as containing cells carrying mutations in the endogenous c-Ha-*ras* oncogene.

There are two possible explanations for the rapid induction of mammary tumors in this system. One would involve an influence of mutation in the exogenous human c-Ha-*ras* gene even though the mutant cell population is a minor component. Thus, unlike mutations in mammary tumors induced by MNU in non-transgenic rats, the mutant cells might be imagined as inducing a malignant phenotype within the majority of non-mutated cells. The other is that the high level of expression of the exogenous human c-Ha-*ras* gene would itself have an effect, assisting mammary carcinogenesis by MNU in rats. Since a point mutation in the last intron of the transgene is known to induce its overexpression (16,32) and the structure of the region is similar in rat and man (33,34), it is possible that an intron point mutation or alteration in the human promoter/enhancer region of the transgene might have influenced expression of the rat endogenous c-Ha-*ras* gene, resulting in enhancement of mammary carcinogenesis. The importance of regulatory elements for c-Ha-*ras* oncogenic activity has been well established using *in vitro* transformation assays and, furthermore, c-Ha-*ras* gene point mutations are not necessary for transformation when the gene is fused with a virus promoter (35).

The results of tumor site and mutational analyses in the current study are not directly comparable with those reported in human c-Ha-*ras* proto-oncogene transgenic mice, because the route of MNU administration differed, with i.v. injection in the rat and i.p. injection in the mouse, the latter inducing forestomach tumors. For an exact comparison of the organ specificity and mutational spectrum between the two species, studies based on the use of the same tumor type, forestomach tumors, which are induced by the same treatment, i.p. injection of MNU, are in progress. An up to 40% incidence of endogenous c-Ha-*ras* gene mutations in forestomach and other site tumors induced by repeated injection of MNU has been noted (36). Our findings of a high incidence of spontaneous skin tumors (unpublished data), which are extremely rare in non-transgenic rats, may provide us with a clue to elucidation of the role of the transduced human c-Ha-*ras* proto-oncogene with regard to enhanced susceptibility to skin carcinogenesis in transgenic mice carrying the same gene.

In conclusion, the human c-Ha-*ras* proto-oncogene transgenic rats reported here show a remarkable enhancement of susceptibility to MNU-induced mammary carcinogenesis, not directly due to transduced human c-Ha-*ras* proto-oncogene and endogenous c-Ha-*ras* gene mutations. Results may greatly facilitate further studies on the analysis of susceptibility to other mammary carcinogens such as DMBA and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, which cause different DNA modifications and have different underlying mechanisms.

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